An essential type I nitroreductase from *Leishmania major* can be used to activate leishmanicidal prodrugs*

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*Running title: *Characterisation of a Leishmanial nitroreductase*

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Background. Leishmaniasis is a tropical disease prevalent in developing countries.

Results: *Leishmania major* type I nitroreductase (LmNTR) metabolises nitroaromatic/quinone substrates, an activity essential for parasite viability.

Conclusion: LmNTR exhibits biochemical characteristics of its bacterial counterparts and its activity is essential for parasite growth.

Significance: LmNTR can be exploited as a prodrug activation enzyme or as a target for inhibitor design in drug development.

SUMMARY

Nitroaromatic prodrugs are used to treat a range of microbial infections with selectivity achieved by specific activation reactions. For trypanosomatid parasites this is mediated by type I nitroreductases (NTRs). Here, we demonstrate that the causative agent of leishmaniasis, *Leishmania major*, expresses a FMN-containing NTR (LmNTR) that metabolises a wide range of substrates and based on electron donor and acceptor preferences may function as a NADH:quinone oxidoreductase. Using gene deletion approaches, we demonstrate that this activity is essential to *L. major* promastigotes, the parasite forms found in the insect vector. Intriguingly, although *LmNTR*$^{+/-}$ heterozygote promastigote parasites could differentiate into infectious form metacyclic cells, these were unable to establish infections in cultured mammalian cells and cause delayed pathology in mice. Further, we exploit the LmNTR activity evaluating a library of nitrobenzylphosphoramide mustards using biochemical and phenotypic screens. We identify a subset of compounds that display significant growth inhibitory properties against the intracellular parasite form found in the mammalian host. The leishmanicidal activity was shown to be LmNTR specific as the *LmNTR*$^{+/-}$ heterozygote promastigotes displayed resistance to the most potent mustards. We conclude that LmNTR can be targeted for drug development by exploiting its prodrug activating property or by designing specific inhibitors to block its endogenous function

Insect transmitted, protozoan parasites belonging to the genus *Leishmania* are responsible for a spectrum of diseases known as leishmaniasis. These infections are endemic in 88 countries around the World with approximately 350 million people living in "at risk" areas (1). Recently, as a result of military activity, population migration, modern medical practices, intravenous drug usage and global warming, leishmaniasis has emerged as a problem in non-endemic areas (2-4).

A vaccine for visceral leishmaniasis is in Phase Is trial but currently drugs are the only option available to treat leishmanial infections. Use of the frontline antimonial-based therapies is problematic as they are toxic, clinical resistance is on the rise and often require medical supervision to administer (5). Recent progress has been made in developing new leishmanicidal agents with several compounds such as amphotericin B, paromomycin and miltefosine

coming to market (1). However, there are issues associated with the use of these as they are expensive and require medical administration with some having teratogenic and other unwanted toxicity problems (6). Against this backdrop, the development of new cost-effective treatments is a priority, but given that leishmaniasis mainly affects people living in developing countries, these infections are not deemed commercially attractive by pharmaceutical companies. As a result, leishmaniasis is largely neglected in terms of drug development (7).

Nitroaromatic compounds encompass a wide range of compounds characterised by at least one nitro-group attached to an aromatic ring (8). They have been used in medicine predominately as antimicrobial agents, but concerns over their mutagenicity have led to many being withdrawn in Europe and USA (9-11). However, it is now apparent that several nitro-based compounds are not as toxic as initially thought (12-14) with retrospective analysis of nitrofurantoin clinical trial data coupled with cost evaluation resulting in calls for the reinstatement of this prodrug as a treatment for uncomplicated urinary tract infections (15). This resurgence of interest has led to several nitroaromatic compounds undergoing evaluation for treatments of infectious organisms including PA-824 and OPC-67683 against *Mycobacterium tuberculosis*, nitazoxanide against *Giardia*, *Cryptosporidium* and hepatitis C (16-18) and fexinidazole against *Trypanosoma brucei* and *Leishmania donovani* (19,20) while others such as SN23862, CB1954 and nifurtimox have emerged as possible anti-cancer therapies (21-23).

Most antimicrobial nitroaromatic compounds function as prodrugs and must undergo activation before producing their cytotoxic activity, a process mediated by nitroreductases (NTRs). Based on oxygen-sensitivity and flavin co-factors, NTRs can be broadly divided into two groups (24). Type I NTRs utilise NAD(P)H as electron donor, transferring reducing equivalents *via* an FMN co-factor, to the substrate in a series of sequential two electron reduction events. This nitroreduction does not involve oxygen and does not result in the production of reactive oxygen species, an activity said to be "oxygen insensitive". In contrast, the type II NTRs contain FAD or FMN as co-factor and catalyse the one electron reduction of the substrates conserved nitro-group to generate a nitro-radical. This radical reacts with oxygen to produce superoxide anions with the subsequent regeneration of the original nitro-compound: type II NTRs is said to be "oxygen sensitive" (25). The difference in NTR distribution is believed to underlie the specificity of most antimicrobial nitroaromatic prodrugs with type I NTRs found mainly in bacterial and absent from most eukarytotes, with a subset of fungi and protozoan parasites being the exceptions (26-28). Although some mammalian enzymes, such as NAD(P)H quinone oxidoreductase 1 and nitric oxide synthase, can mediate two electron reduction reactions under aerobic conditions, type II NTR activities predominate in most cell types (29).

The aim of this study was to characterise the type I NTR expressed by *L. major*, the pathogen responsible for cutaneous leishmaniasis, and analyse the role this enzyme plays in the parasite. Utilising the activity, we then conducted biochemical and phenotypic screens using a library of nitrobenzylphosphoramide mustard (NBPM) compounds to identify potential leishmanicidal agents targeting the intracellular mammalian form of the pathogen.

EXPERIMENTAL PROCEDURES

Compounds. Nitrofurazone, nitrofurantoin, CB1954, coenzyme Q1 and duroquinone were purchased from Sigma Aldrich. Benznidazole and nifurtimox were provided by Simon Croft (London School of Hygiene and Tropical Medicine) and metronidazole was a gift from Nubia Boechat (Far Manguinhos, Rio de Janeiro). The NBPM structures (Tables 1 and 2) were fully characterized using NMR and MS and their purity judged to be >90 %: most were >95 % based on LC-MS analysis (30-33).

Parasite culture and genetic manipulation. *L. major* (MHOM/IL/80/Friedlin) promastigote form parasites were grown at 27 ^{o}C in M199 medium (Life Technologies) supplemented with 4 mM sodium bicarbonate, 40 mM HEPES pH7.4, 0.1 mM adenine, 0.005 % (w/v) haemin, 2.5 U mL^{-1} penicillin, 25 μg mL^{-1} streptomycin and 20% (v/v) foetal calf serum. Transformed parasites were grown in this medium containing G418 (20 μg mL^{-1} on agar plates, 40 μg mL^{-1} in broth), blasticidin (10 μg mL^{-1}) or puromycin (20

μg mL^{-1}). *L. major* metacyclic form parasites were harvested from promastigote cultures in the stationary phase of growth (7-10 days old cultures) following agglutination of promastigote parasites with peanut lectin (Sigma Aldrich) (34). The metacyclics were used to infect differentiated human acute monocytic leukemia (THP-1) cells at a ratio of 20 parasites per mammalian cell. The *L. major* infected monolayers were incubated overnight at 37 ^{o}C under a 5 % (v/v) CO_2 atmosphere in mammalian growth medium then washed with RPMI-1640, to remove residual parasites. *L. major* amastigote parasites were maintained in differentiated THP-1 cells at 37 ^{o}C under a 5 % (v/v) CO_2 atmosphere in mammalian growth medium.

For DNA transfection, *L. major* promastigotes (2 x 10^7) in the logarithmic phase of growth were pelleted, resuspended in 100 μL Human T-cell Nucleofector® solution (Lonza AG) containing 5 μg of purified DNA and electroporated using program U-033 on the Nucleofector™ (Lonza AG). Cells were then transferred to M199 growth medium and allowed to recover for 20-24 hours at 27 ^{o}C. Parasites were pelleted, resuspended in 100 μL promastigote growth medium then spread onto promastigote growth medium solidified with 1% (w/v) agar containing 1.2 μg mL^{-1} biopterin (Sigma-Aldrich) and the appropriate selective agent. The plates were incubated at 27 ^{o}C until colonies appear. Colonies were then transferred into promastigote growth medium containing the appropriate selective agent and grown as described above.

Mammalian cell culturing. The human acute monocytic leukemia cell line (THP-1) was grown at 37 ^{o}C under a 5 % (v/v) CO_2 atmosphere in RPMI-1640 medium (PAA Laboratories Ltd) supplemented with 2 mM pyruvate, 2 mM sodium glutamate, 2.5 U mL^{-1} penicillin and 2.5 μg mL^{-1} streptomycin, 20 mM HEPES pH 7.4 and 10% (v/v) foetal calf serum. Differentiation of THP-1 towards macrophage-like cells was carried out using phorbol 12-myristate 13-acetate (20 ng mL^{-1}) (PMA) (Sigma-Aldrich) (35).

In vivo studies. All animal experiments were conducted under licence in accordance with UK Home Office regulations. Female BALB/c mice were infected by subcutaneous injection of 2×10^7 purified metacyclic parasites in 100 μL RPMI medium without serum into their shaved rump. Mice were monitored for lesion pathology and when observed, the diameter of each lesion was measured in two dimensions at a rectangular angle with digital callipers (Jencons Scientific Ltd.) and the mean diameter calculated. Three mice were examined for each treatment.

Antiproliferative assays. All assays were performed in a 96-well plate format. *L. major* promastigote parasites (5 x 10^5 mL^{-1}) or differentiated THP-1 cells (2.5 x 10^4 mL^{-1}) were seeded in 200 μL growth medium containing different concentrations of nitroaromatic agent. After incubation at 27 ^{o}C for 5 days (*L. major*) or at 37 ^{o}C for 3 days (THP-1), 2.5 μg resazurin (20 μL of 0.125 μg mL^{-1} stock in phosphate buffer saline) was added to each well and the plates incubated for a further 8-16 hours. Cell densities were determined by monitoring the fluorescence of each culture using a Gemini Fluorescent Plate Reader (Molecular Devices (UK) Ltd, Wokingham, UK) at an excitation wavelength of 530 nm, emission wavelength of 585 nm and a filter cut off at 550 nm, and the drug concentration that inhibits cell growth by 50 % (IC_{50}) established.

Growth inhibition of luciferase expressing *L. major* amastigotes was monitored as follows. THP-1 cells seeded at 2.5 x 10^4 mL^{-1} in 200 μL in growth medium containing PMA (20 ng mL^{-1}) were incubated at 37 ^{o}C in a 5 % (v/v) CO_2 atmosphere for 3 days. Macrophage monolayers were copiously washed with mammalian growth medium then infected with purified *L. major* metacyclic cells (5 x 10^5 cells mL^{-1}) resuspended in 200 μL mammalian growth medium. Following incubation overnight at 37 °C in a 5 % (v/v) CO_2 atmosphere, the cultures were washed twice in growth medium to remove non-internalised parasites and the supernatant replaced with fresh growth medium containing drug. Drug-treated infections were incubated for a further 3 days at 37 ^{o}C under a 5 % (v/v) CO_2. The growth medium was then removed and the cells lysed in 50 μL cell culture lysis reagent (Promega). Activity was then measured using the luciferase assay system (Promega) and light emission measured on a ß-plate counter (Perkin Elmer). The luminescence is proportional to the number of live cells. The IC_{50} value for each compound was then established.

Plasmids. The integrative *L. major* luciferase vector pLmRIX-luc was generated by sequentially cloning the *L. major* 5' spacer/promoter rRNA and 3' spacer rRNA regions either side of an expression cassette containing the luciferase reporter and neomycin resistance genes. The *TcMPX* polypyrimidine tract/splice leader addition site sequence was inserted between the *L. major* 5' spacer/promoter rRNA and the luciferase gene to provide the genetic elements required for processing the reporter mRNA transcript. The resultant construct, pLmRIX-Luc, was linearized prior to electroporation into *L. major* promastigotes.

The vectors used to delete *LmNTR*, pKO-*LmNTR*-PAC and pKO-*LmNTR*-BLA, were generated by sequentially cloning *LmNTR* flanking sequences either side of a puromycin or blasticidin resistance cassette (see Fig. 3). The constructs were linearized then electroporated into *L. major* promastigotes.

A DNA fragment encoding for the catalytic domain of *L. major NTR* (*LmNTR*) was amplified from genomic DNA with the primers *ggatcc*CTCGACGCCGTCGAGGCCGTCG and *aagctt*CTAGAACTTGTTCCACCGCAC; lower-case italics correspond to restriction sites incorporated into the primers to facilitate cloning. The fragment was digested with *Bam*HI/*Hin*dIII then cloned into the corresponding sites of the vector pTrcHisC (Invitrogen) to form the plasmid pTrcHisC-LmNTR. Site-directed mutagenesis was carried out using a Stratagene QuikChange mutagenesis kit (Agilent Technologies), with pTrcHisC-LmNTR as template. Amplifications were performed in accordance with the manufacturer's instructions using primers (Eurofins) to generate the desired mutation. The forward primer sequence was R96A (GCCGTCGTGCGCGACGCGTGGACGTGCC GGCAG). The relevant substitution site, incorporating the required base change, is underlined.

Protein purification. An overnight culture of *E. coli* BL21(+) containing the expression plasmid was diluted 1:50 in NZCYM (Sigma Aldrich) medium containing 100 μg mL^{-1} ampicillin and grown for 2-4 h at 37 °C. The culture was transferred to 16 °C for 30 min then protein expression induced by addition of isopropyl-β-d-thiogalactopyranoside to a final concentration of 1 mM. The culture was then incubated overnight at 16 ^{o}C. The cells were harvested then lysed in Buffer A (50 mM NaH_2PO_4, pH 7.8; 500 mM NaCl) containing 1 mg mL^{-1} lysozyme, 10 μg mL^{-1} , DNase, 10 μg mL^{-1} RNase, 1 % (w/v) CHAPS and a cocktail of protease inhibitors (Roche). The clarified supernatant was then applied to a pre-packed nickel-nitrilotriacetic acid (Qiagen) column and the column copiously washed with Buffer A and with Buffer A containing 50 mM and 100 mM imidazole. Recombinant protein was eluted off the column using Buffer A supplemented with 500 mM imidazole and 1 % (w/v) CHAPS. Glycerol was then added to a final volume of 20 % (v/v) and aliquots stored at -80 ^{o}C. Protein concentration was determined by the BCA assay system (Thermo Fisher Scientific, Cramlington, UK) and purity level confirmed by SDS/PAGE.

Enzyme activity. LmNTR activity was measured spectrophotometrically using several electron acceptors as substrate. A standard reaction (1 mL) containing 50 mM Tris-Cl pH7.5, 100 μM NADH and 100 μM electron acceptor was incubated at room temperature for 5 min. The background reaction rate was determined and the assay initiated by addition of the LmNTR (35 μg). For reactions containing nitroimidazoles, nitrobenzylphosphoramides or most quinones, activity was measured by following the change in absorbance at 340 nm corresponding to NADH oxidation (ε = 6,200 M^{-1} cm^{-1}) while for assays involving nitrofurans, the direct reduction of the substrate itself was monitored at 435 nm for nifurtimox (ε = 18,000 M^{-1} cm^{-1}) or at 400 nm for nitrofurazone and nitrofurantoin (ε = 12,000 and 15,000 M^{-1} cm^{-1} respectively; (36)). For CB1954, activity was monitored by following the change in absorbance at 420 nm, corresponding to production of the hydroxylamine (ε = 1,200 M^{-1} cm^{-1}: (37)). Data was evaluated by non-linear regression analysis using GraphPad Prism 5 (GraphPad Software).

Flavin characterization. The flavin co-factor bound to LmNTR was established by determining the fluorescence spectrum in acidic and neutral buffers (36,38). Purified protein was desalted and boiled for 5 min. In a total volume of 100 μL, clarified supernatants (90 μL) containing 0.5 mg NTR were mixed with 10 μL 50 mM NaH_2PO_4 pH7.6 or 1 M HCl (final pH = 2.2). The fluorescence profile for each treatment was then determined using a Gemini Fluorescent

Plate Reader (Molecular Devices Ltd) with an excitation λ= 450 nm and an emission λ= 480-600 nm. The resultant patterns were compared with profiles obtained using FMN and FAD standards

RESULTS

***Leishmania major* expresses a functional type I nitroreductase.** Analysis of the *L. major* genome database identified a 972 bp open reading frame located on chromosome 5 with potential to encode for a 34.7 kDa enzyme related to the type I nitroreductase family of proteins (LmNTR; GenBank accession no. XP_001687543). Full length LmNTR is 90 % identical to homologues present in *L. mexicana* (CBZ23423) and *L. infantum* (CAM65376) NTRs), 75 % to *L. braziliensis* NTR (CAM36899)) and has 50 % identity to the trypanosomal enzymes TcNTR (XP_810645) and TbNTR (XP_846343). When compared to bacterial counterparts such as *E. coli* nfsB (NP_415110) and *Bacillus cereus* NAD(P)H nitroreductase (NP_832770) the highest level of sequence identity was around 20 %. Based on sequence, LmNTR can be divided into two regions. The amino terminal portion (residues 1-86), absent from prokaryotic sequences, is predicted by SignalP, PSORT II and iPSORT algorithms to contain a mitochondrial targeting signal (data not shown) while the remainder of the enzyme (residues 87-323) constitutes the catalytic domain.

To evaluate whether LmNTR could function as a nitroreductase, the DNA sequence encoding for the putative catalytic domain (residues 87-323) was expressed as a HIS-tagged protein in *E. coli*: attempts to express full length recombinant LmNTR failed to generate soluble protein. The recombinant enzyme could be readily purified after one round of affinity chromatography with elutions containing the purified HIS-tagged LmNTR having a yellow colouration, indicative of a flavoprotein (Fig. 1A). To identify the nature of the flavin and investigate NTR/co-factor interaction, recombinant LmNTR was boiled and the fluorescence profile of clarified supernatants under neutral and acidic pHs analysed (36,38) (Fig. 1B). Denaturation resulted in release of the flavin from the enzyme, indicating that the co-factor had a non-covalent association with the protein backbone (39-41), while the fluorescence profiles under neutral and acidic pHs identified FMN as the flavin moiety (Fig. 1B): at neutral pH, and excitation at 450 nm, flavin derived from the parasite enzyme had a peak emission around 560 nm, a signal quenched in acidic buffers, typical of FMN and distinct from FAD. In the trypanosomal enzyme TcNTR an arginine located in the RX[S/T/A]X[R/K] motif has been shown to play a key role in co-factor binding with this region and residue conserved in LmNTR (42). To evaluate the importance of this arginine to LmNTR activity it was converted to alanine (R96A). Expression of the HIS-tagged mutant protein in *E. coli* resulted in recombinant protein but at levels considerably lower than that obtained with the wild type enzyme. The mutated enzyme remained in insoluble fractions despite several attempts to optimise expression and purification conditions (data not shown).

To investigate its substrate specificity, HIS-tagged LmNTR activity was monitored under aerobic conditions by following NADH or NADPH oxidation at 340 nm, or, when a compound's absorbance spectra precluded this, by following reduction of the substrate itself (Fig. 2A). This demonstrated that the recombinant leishmanial enzyme functioned as typical type I NTR, able to catalyze the reduction of a wide range of nitroaromatics and quinones in an oxygen insensitive fashion (Table 3). When the specificity constants (k_{cat}/K_M) generated by these substrate were compared LmNTR showed a preference for quinone compounds: the k_{cat}/K_M values exhibited by HIS-tagged LmNTR toward the ubiquinone 5 (2.0×10^4) was an order of magnitude greater than for CB1954 (4.6×10^3) and benznidazole (2.5×10^3). Interestingly, recombinant LmNTR could use both NADPH and NADH as electron donors although when using fixed concentrations of benznidazole (100 μM) and NADPH or NADH (100 μM) activity values indicated a preference for NADH: under these conditions LmNTR had a specific activity of 110 nmol NADH oxidised min^{-1} mg^{-1} as compared to 50 nmol NADPH oxidised min^{-1} mg^{-1}. Using benznidazole (100 μM) as substrate, HIS-tagged LmNTR had an apparent *Km* for NADH of 76 μM, similar to the values reported for TbNTR and TcNTR (71 and 86 μM, respectively) (42).

To examine how recombinant LmNTR interacts with NADH, assays were carried out using various concentrations of reductant against a fixed concentration of benznidazole (Fig. 2B).

For this substrate, double-reciprocal plots were linear at all concentrations of electron acceptor, with the slopes remaining parallel. When the converse experiments were performed using a fixed concentration of NADH and various amounts of benznidazole, a similar pattern of parallel slopes were observed (Fig. 2B). These patterns are characteristic of a ping-pong mechanism of kinetics, typical for oxidoreductase cascades. However, when benznidazole or NADH levels were above 200 μM non-competitive substrate inhibition was observed indicating that this mechanism of kinetics only occurs over a limited electron donor/acceptor concentration range (Fig. 2C).

Functional analysis of LmNTR in *L. major*. To investigate whether the leishmanial type I NTR can activate nitroaromatic drugs in the parasite itself, we developed *L. major LmNTR* heterozygotes and then examined the susceptibility of the cells to nifurtimox. DNA fragments corresponding to the regions immediately upstream and downstream of *LmNTR* were amplified from genomic DNA and cloned sequentially either side of cassettes containing puromycin or blasticidin resistance markers. The constructs were linearised and the purified fragments transformed into *L. major* promastigotes with clones selected on agar plates containing puromycin or blasticidin. Southern hybridisation of genomic DNA showed that one *LmNTR* allele could be disrupted generating heterozygous parasites (*LmNTR*$^{+/-}$*PAC* or *LmNTR*$^{+/-}$*BLA*) (Fig. 3), with no obvious effect on parasite growth or ability to differentiate into infective metacyclic forms (Fig. 4A). To evaluate whether reduction of the *LmNTR* copy number affected resistance to nitroheterocyclic drugs, *LmNTR*$^{+/-}$*BLA* promastigote parasites were grown in the presence of nifurtimox (Fig. 4B). From the resultant dose response curves, the IC_{50} value of the heterozygote line was shown to be approximately 2-fold higher than wild type parasites: *L. major* wild type cells exhibited an IC_{50} value of 6.28 ± 0.04 μM as compared to 11.50 ± 0.60 μM for the *LmNTR*$^{+/-}$ line.

Attempts (16 independent transformations) to generate *LmNTR* null mutant lines failed, leading us to speculate that the protein is essential to promastigote form parasites. To confirm this we constructed a range of pTEX- and pIR-SAT1-based episomal and integrative complementation vectors designed to facilitate expression of LmNTR or variants tagged at the carboxyl terminal with an epitope derived from the human c-myc protein. Attempts (20 independent transformations) to generate parasites lines containing an ectopic copy of *LmNTR* failed, leading us to postulate that expression of elevated NTR levels was also deleterious to *L. major* promastigotes. Even when using "amastigote-specific" integrative vectors, where *LmNTR* expression was placed under the control of the well characterised *Leishmania* regulatory genetic element (the amastin 3' UTR (43)), no recombinant parasites were obtained: the "amastigote-specific" expression system was validated using constructs expressing luciferase where the reporter activity under the control of the amastin 3' UTR was only 2-10 fold above background while in the absence of this regulatory genetic element reporter activity >1000 fold higher (data not shown). This toxicity may be in part due to LmNTRs amino terminal. To facilitate localisation, attempts (6 independent transformations) to generate *L. major* promastigotes expressing the amino terminal extension (residues 1-85) of LmNTR fused at its carboxyl terminal with GFP or RFP failed despite using episomal or integrative expression vectors (data not shown).

To aid the characterisation of *L. major LmNTR* heterozygotes, *LmNTR*$^{+/-}$*BLA* cells were transformed with a DNA fragment that integrates into the *L. major* rRNA promoter/spacer region and facilitates expression of luciferase. Following selection, the luminescence activity of clonal parasite extracts was shown to be $>10^6$ fold above background, comparable to lysates generated from luciferase expressing wild type cells. These parasites also exhibit promastigote growth and metacyclogenesis rates equivalent to wild type/non-luciferase expressing *LmNTR* heterozygotes (data not shown). Metacyclic form parasites from luciferase tagged *LmNTR*$^{+/-}$ heterozygote and wild type promastigote cultures in the stationary phase of growth were then purified and used to infect differentiated THP-1 macrophage cells. Over the following 4 days, total cell extracts were generated from parasite/mammalian cultures and the luciferase activity determined (Fig. 5A). After correcting for background luminescence (lysed, uninfected macrophages), the reporter signal generated from tagged wild type amastigotes increased, reaching a plateau by day 4 post-infection. In contrast,

LmNTR$^{+/-}$ heterozygote cultures exhibited luminescence values equivalent to background. This indicates that disruption of one *LmNTR* allele results in haploid insufficiency such that *L. major LmNTR* heterozygote amastigotes are unable to establish themselves in tissue cultured mammalian macrophages.

To determine whether the *in vitro* amastigote proliferation defect translates to problems with *in vivo* growth, BALB/c mice were infected with purified wild type or *LmNTR*$^{+/-}$ heterozygote *L. major* metacyclic cells. Over a 97 day period, the presence and size of any lesion was monitored (Fig. 5B). All 3 mice infected with wild type parasites developed lesions by day 31 with the lesions gradually increasing in size: all mice were euthanized at day 51. Interestingly, all mice infected with *LmNTR*$^{+/-}$ heterozygote cells presented no signs of lesion until day 98, where single mouse developed a lesion, with the remaining mice developing pathologies 24 days latter (day 122). In all cases the lesion size gradually increased until day 128 when all mice were euthanized. The altered kinetics in pathology development strongly indicates that LmNTR function is important in establishing a *L. major* infection *in vivo* with the heterozygote line struggling to grow as amastigote form parasites in BALB/c mice.

Exploiting the pro-drug activating properties of the *Leishmania* type I NTR. We have previously shown that NBPM compounds are effective NTR-activated trypanocidal agents (36). As an initial screening strategy to determine the potential for exploiting LmNTR activity in prodrug activation, we determined whether HIS-tagged LmNTR displayed activity toward a library of NBPMs by monitoring the change in absorbance at 340 nm, corresponding to NADH oxidation (Fig. 6A). In total, 22 compounds were screened: 8 structures where either a *p*-nitrobenzyl group or an *o*- nitrophenylacetamido group is attached to cyclophosphamide (Table 1) and 14 structures where phosphoramide mustard is linked to a nitrobenzyl group with various substituents (Table 2). Based on the preliminary biochemical screen, the compounds could be divided into those that were not LmNTR substrates (activity <20 nmol NADH oxidised min^{-1} mg^{-1}), those that stimulated a moderate activity (between 20-50 nmol NADH oxidised min^{-1} mg^{-1}) and those that were readily metabolised by the parasite enzyme (>50 nmol NADH oxidised min^{-1} mg^{-1}). Using this as a crude measure, 3 cyclic NBPMs (LH5, 6 and 12) were evaluated as moderate substrates while the remaining 5 showed little/no activity. In contrast, most of the acyclic structures were metabolised by recombinant LmNTR with 6 (LH27, 31-34 and 37) showing high levels of activity. Of those substrates generating the higher activities, 5 contained at least one halogen linked to the nitro-substituted benzene ring.

To determine whether there was a correlation between biochemical activity and parasite killing, all NBPMs were initially screened for leishmanicidal activity against *L. major* promastigote and amastigote forms. Out of the 22 compounds, 15 were shown not to affect growth of the insect stage pathogen at 30 μM with all except one (LH19) also having no effect on amastigote growth at a concentration of 10 μM (Table 4). For the remaining leishmanicidal compounds, growth inhibition assays were performed to determine their IC_{50} values (Table 4). Many of these displayed appreciable leishmanicidal properties, with 5 NBPMs (LH31-34 and 37) having significant activity (IC_{50}'s <10 μM) against both parasite forms. These 5 compounds correspond to structures previously designated "good" LmNTR substrates (Fig. 6).

The 8 compounds identified as having appreciable leishmanical activity were assayed for cytotoxicity against differentiated THP-1 cells from which the selective index (SI) (IC_{50} against the mammalian line/IC_{50} against the amastigote parasite) was then determined (Table 4). In all cases, no toxicity to the mammalian line was observed at concentrations up to 100 μM. For three of the halogenated compounds identified as being preferred LmNTR substrates and having anti-parasitic activity against both *L. major* forms (LH32-34), SI values against amastigote parasites of >100 were observed.

To demonstrate that NTR plays a role in NBPM prodrug activation within the parasite itself, the susceptibility of *L. major LmNTR*$^{+/-}$ *BLA* heterozygote promastigotes to LH33 and LH34 was investigated (Fig. 6B). For both compounds, cells with reduced levels of the nitroreductase were up to 3-fold more resistant to the agent than wild type controls: *L. major* wild type cells exhibited IC_{50} values of 4.73 ± 0.29 and 3.70 ± 0.40 μM toward LH33 and LH34,

respectively with *LmNTR*$^{+/-}$ heterozygote cells displaying values of 7.66 ± 0.23 and 12.14 ± 0.84 μM against these two compounds.

Based on our findings, NBPMs that contain the phosphoramide mustard as part of an acyclic structure and have halogen substituents on the nitrobenzyl ring are the most readily metabolised by the LmNTR enzyme and represent the most potent agents against both *L. major* promastigotes and amastigotes. Some (LH32, 33 and 33) have IC_{50} values of 1 μM or less against the intracellular stage without inducing mammalian cell toxicity.

DISCUSSION

Activation of most antimicrobial nitroaromatic prodrugs occurs through reactions catalysed by type I NTRs (24,26). This group of enzymes were believed to be restricted to bacteria but it is now apparent that they are also expressed by several 'lower' eukaryotes including fungi and protozoan parasites (20,26-28). Interestingly, type I NTRs appear to be absent from 'higher' eukaryotes including humans, and it is believed that this difference in the enzymes' distribution underlies the antimicrobial selectivity of many nitroaromatic prodrugs. Here, we demonstrate that *L. major* expresses a type I NTR that possesses many biochemical characteristics displayed by its bacterial counterparts. Functional studies revealed that this activity is essential for the growth of parasite forms found in the insect vector and extremely important to the intracellular forms found in mammalian cells, indicating that this prodrug activating enzyme is a drug target in its own right.

The activity of all type I NTRs, irrespective of their origin, is dependent on FMN with the enzyme/flavin interaction occurring *via* a non-covalent linkage, features shared by LmNTR (Fig. 1): HIS-tagged LmNTR activity was associated with yellow coloured fractions, boiling released the associated co-factor from the protein backbone with clarified supernatants exhibiting fluorescence profiles under acidic and neutral pH's identical to that observed with FMN (Fig. 1). For the leishmanial enzyme, the co-factor acts an intermediary, accepting reducing equivalents derived preferentially from NADH then denoting these to a range of nitroaromatic and quinone based substrates (Table 3). While catalysing this transfer, HIS-tagged LmNTR displays a ping-pong type of kinetics, typical of oxidoreductases (Fig. 2). In TcNTR, co-factor binding is mediated by a specific arginine residue at position 90 present in a conserved motif. This amino acid (R96 in LmNTR) plus the adjacent region is present in NTR from *L. major*. Attempts to express a mutated version of the leishmanial enzyme (R96A) failed to generate soluble, active protein suggesting that the non-covalent FMN binding to the protein backbone may be important in the correct folding of LmNTR.

Based on its substrate preferences (Table 3) and subcellular localisation, as inferred from its sequence, LmNTR may function as a mitochondrial NADH:quinone oxidoreductase. In the mitochondria of most eukaryotes, oxidation of NADH to NAD^+ is normally mediated by complex I of the electron transport chain. This activity serves to translocate protons across the organelle's inner membrane with the concomitant reduction of ubiquinone to ubiquinol. Ubiquinol then drives the cytochrome-dependent respiratory chains that help to form the proton motive force which ultimately leads to ATP synthesis. In contrast to bloodstream dwelling trypanosomes all replicative *Leishmania* forms express functional cytochrome-dependent electron transport chains suggesting that energy production occurs *via* the well documented route within these parasites (44-46). However, the role played by the *Leishmania* complex I in driving these cascades is unclear. Inhibitor studies indicate that these protozoa possess an atypical complex I with bioinformatic studies revealing a subunit composition distinct from that found in other eukaryotic organisms (44,47). As LmNTR appears to fulfil an analogous role to the leishmanial complex I, with both functioning as NADH:quinone oxidoreductases, these may act in concert to help maintain the NADH/NAD^+ balance within the parasite mitochondrion.

The endogenous function of LmNTR is essential to replicating *L. major*: *LmNTR* could not be deleted from non-infectious promastigote parasites with the heterozygotes unable to establish an infection in cultured macrophages and struggled to generate lesion pathology in mice (Fig. 5). This is similar to the situation in *L. donovani* but distinct from that observed in trypanosomes where the essential nature of NTR is only apparent in the replicating forms present

in the mammalian host (26,48). As we were unable to express elevated levels of LmNTR in *L. major* heterozygote and wild type promastigotes the appropriate complementation experiments could not be performed. Intriguingly, complementation studies could be conducted using *L. donovani* promastigotes (48): *L. donovani NTR* null mutants could be generated only in the presence of an ectopic copy of *L. major NTR*. The deleterious effect of expressing LmNTR at elevated levels may not be due to the enzymatic activity of the protein itself but could reflect an inherent problem associated with the amino terminal extension. Therefore, when expressed at levels higher than that found in wild type (and heterozygote) the LmNTR extension may interfere with mitochondrial transporter function or could interact with other essential cellular components thereby inhibiting their activities.

Nitrobenzylphosphoramide mustards are a novel class of compounds incorporating chemical motifs present in several DNA alkylating, anti-cancer agents (30-33). They contain a nitrogen mustard moiety coupled to a nitrobenzyl ring *via* a phosphoramide linker and are designed to function as prodrugs specifically tailored to undergo activation in reactions catalysed by type I NTRs. Here, we performed a structure activity relationship (SAR) on a library of NBPMs employing biochemical and leishmanicdal screens (Fig. 6 and Table 4). When using a group of NBPMs where the phosphoramide linker was part of a cyclic structure (Table 1), recombinant LmNTR protein was shown to metabolise some (3 out of 8) at reasonable rates but this failed to translate into an anti-parasitic activity. Screens involving NBPMs where the phosphoramide was part of a linearised linker (Table 2) yielded similar results for 6 out of 14 compounds with the remaining NBPMs being effective LmNTR substrates and displaying leishmanicidal effects. When comparing the anti-parasitic properties exhibited by the 8 most potent NBPMs against the two replicative *L. major* life cycles stages, intracellular amastigote parasites were generally more susceptible to the agent under study than the promastigote form: LH33 had IC_{50}'s of 5.88 ± 0.28 and 0.21 ± 0.07 μM against promastigote and amastigote form parasites, respectively, while LH34 exhibited values of 3.10 ± 0.28 and 0.77 ± 0.12 μM. To conclusively demonstrate that the LmNTR plays a role in NBPM activation in the parasite itself, the susceptibility of *L. major LmNTR*$^{+/-}$ heterozygous cells to LH33 and LH34 was evaluated. In both cases, reduction of the *LmNTR* gene copy number resulted in a resistance phenotype in agreement with observation made using recombinant trypanosomes and *L. donovani* towards other nitroaromatics (Fig. 6) (26,48). As mammalian cells lack a type I NTR activity, they should be less susceptible to prodrugs that rely on this mechanism of activation. When the 8 most potent leishmanicidal NBPMs were screened against the macrophage line in which the intracellular parasites were cultured, no cytotoxicity was observed at values up to 100 μM) (Table 4). In terms of relative toxicity values, the 2 agents showing the highest potency against amastigote cells were >476 (LH33) and >143 (LH34) more toxic to the parasite than this particular mammalian line.

We have now shown that *L. major* expresses a type I NTR which in the related trypanosomal parasites activates the clinically used prodrugs nifurtimox and benznidazole (26,49,50). The leishmanial enzyme displays characteristics typical of this group of oxidoreductase being able to non-covalently bind with FMN and, through a ping-pong mechanism, utilises NADH to reduce a wide range of nitroaromatic and quinones. We exploited this activity and identified several leishmanicidal NBPM compounds that have little/no cytotoxicity in mammalian cells. Based on substrate preference, the *L. major* type I NTRs can be regarded as NADH:quinone oxidoreductase, with this activity being important to the replicative parasite forms found in the insect and mammalian host. Therefore, our data indicate that the leishmanial enzyme can be used in drug development in two ways, either as an activator of new, nitroaromatic or quinone-based prodrugs and through the use of inhibitors targeting this essential activity. Additionally, these two approaches could be complementary: an *L. major* strain showing resistance to drugs developed to inhibit LmNTR could theoretically be sensitive or even hypersensitive to prodrugs activated by this leishmanial enzyme, and *vice versa*.

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FOOTNOTES

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The abbreviations used are: BLA, blasticidin resistance gene cassette; IC_{50}, compound concentration that inhibits cell growth by 50 %; LmNTR, *L. major* type I nitroreductase; NBPM, nitrobenzylphosphoramide mustard; NTR, type I nitroreductase; PAC, puromycin resistance gene cassette; PMA, phorbol 12-myristate 13-acetate; rRNA, ribosomal RNA; TbNTR, *T. brucei* type I nitroreductase; TcNTR, *T. cruzi* type I nitroreductase; UTR, untranslated region

FIGURE LEGENDS

Figure 1 Leishmanial NTRs contain FMN as a cofactor. (A) Coomassie-stained SDS-PAGE gel (10%) containing purified, recombinant LmNTR (lane 2). Lane 1, size standards. (B) Fluorescence spectra of FMN and FAD (both 50 μM) and of supernatant from boiled and purified recombinant LmNTR (0.5 mg) at pH 7.6 (solid line) and pH 2.2 (dashed line) with excitation at 450 nm and emission between 480 and 600 nm. All the fluorescence analyses were carried out in triplicate; the profiles are derived from the mean values.

Figure 2. Investigation of the kinetic properties of leishmanial type I nitroreductase toward benznidazole. (A) Postulated scheme for the *L. major* type I nitroreductases (NTR) mediated reduction of benznidazole (BNZ) by the using NADH as an electron donor. "Red" represents the reduced form and "oxid" represents the oxidized form of benznidazole. The oxidized (FMN) and reduced (FMNH2) forms of the flavin cofactor are indicated. (B, left) Interaction of LmNTR with NADH (reaction I). Activity was assayed by monitoring the oxidation of NADH (30 to 100 μM) in the presence of benznidazole (50 μM [▲], 75 μM [●], and 100 μM [■]) and HIS-tagged LmNTR (35 μg). (Right) Interaction of LmNTR with benznidazole (reaction II). Activity was assayed by monitoring the oxidation of NADH (70 μM [▲], 80 μM [●], and 100 μM [■]) in the presence of benznidazole (20 to 100 μM) and HIS-tagged LmNTR (35 μg). (C) Inhibition of LmNTR activity by high concentrations of NADH or benznidazole. In reaction I, a fixed concentration of benznidazole (100 μM) was reduced by HIS-tagged LmNTR (35 μg) using various concentrations of NADH (50 to 400 μM). At high NADH levels (>200 μM), substrate inhibition was observed. In reaction II, a fixed concentration of NADH (100 μM) was oxidized by HIS-tagged LmNTR (35 μg) using various concentrations of benznidazole (50 to 400 μM). At benznidazole concentrations above 200 μM, substrate inhibition was observed. All assays were initiated by the addition of the parasite enzyme. LmNTR activity is expressed as μmol NADH oxidized min^{-1} mg $protein^{-1}$, while [NADH] and [benznidazole] are expressed in μM.

Figure 3. Disruption of *LmNTR* in *L. major*. (A) Diagram of the *LmNTR* alleles and the effects of gene disruption. 5' and 3' untranslated regions (dashed line) immediately upstream and downstream of *LmNTR* (black box) were amplified and cloned sequentially either side of a puromycin (*pac*; dark grey box) or blasticidin (*bla*; white box) cassette containing *T.brucei* tubulin intergenic elements required for processing of mRNA (light grey boxes). The position of the predicted *Apa*I (A) sites plus the band sizes (in kbp) obtained after hybridisation are shown. (B) Autoradiographs of ApaI digested genomic DNA from *L, major* (lane 1), $LmNTR^{+/-}PAC$ and $LmNTR^{+/-}BLA$ heterozygous clones (lanes 2 and 3 respectively). Blots were hybridized with labelled 5' untranslated region of *LmNTR*. Sizes given are in kbp.

Figure 4. Promastigote growth and metacyclogenesis of the *L. major LmNTR* heterozygous line. (A) *L. major* wild type (square; solid line) and $LmNTR^{+/-}BLA$ heterozygote (circle; dotted line) promastigote parasites growth was monitored until cultures were in the stationary phase of growth (time = 240 h). At 120 h onward, the number of metacyclic form parasites in wild type (triangle; solid line) and $LmNTR^{+/-}BLA$ heterozygote (cross; dotted line) promastigote culture was determined following purification by agglutination. The data is expressed as % metacyclics load in the total *L. major* population. All curves shown are derived from a single data set and are representative of experiments performed in triplicate. (B). Dose response curves of nifurtimox on *L. major* wild type (wt) and $LmNTR^{+/-}BLA$ heterozygote ($NTR^{+/-}$) promastigotes. Data are means from four experiments ± standard deviations, and the differences in susceptibility were statistically significant ($P < 0.01$), as assessed by Student's *t* test.

Figure 5. Disruption of a single LmNTR allele affects infectivity. (A) Purified *L. major* wild type (wt) and $LmNTR^{+/-}BLA$ heterozygote ($NTR^{+/-}$) metacyclic form parasites engineered to express luciferase were used to infect differentiated THP-1 cells. Over a 4 day post infection period, extracts

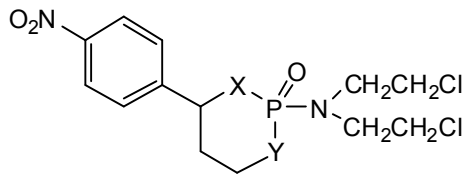
were generated from each cell line and the luciferase activity determined. Following background correction, the luciferase activity was plotted against time. All curves shown are derived from a single data set and are representative of experiments performed in triplicate. (B) Purified *L. major* wild type (wt) and *LmNTR*$^{+/-}$BLA heterozygote (*NTR*$^{+/-}$) metacyclic form parasites were inoculated into the rump of BALB/c mice. Periodically, the diameter of the lesion was measured. For each cell line, three mice were infected and the data is expressed as the mean lesion size (in mm) ± standard deviation. For the heterozygote infections, the lesion sizes on days 98 and 114 are derived from a single mouse as the wounds present on other animals could not be accurately determined.

Figure 6. Evaluating nitrobenzylphosphoramide mustards as LmNTR substrates. (A) The activity of purified HIS-tagged LmNTR was assessed by using various NBPMs (100 μM) as substrate at a fixed concentration of NADH (100 μM). The values shown are the means of data from three experiments ± standard deviations. LmNTR activity was deemed to be high if it was >50 nmol NADH oxidized min^{-1} mg^{-1} (dotted line). The activity obtained when using nifurtimox (NFX) as a substrate is also shown. (B). Dose response curves of *L. major* wild type (wt) and *LmNTR*$^{+/-}$*BLA* heterozygote (*NTR*$^{+/-}$) promastigotes to LH33 and LH34. Data are means from four experiments ± standard deviations.

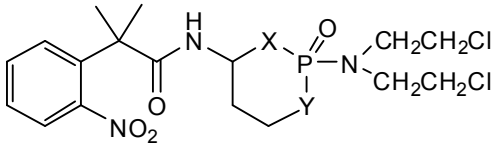
TABLES

Table 1: Structure of cyclic nitrobenzylphosphoramide mustards.

A

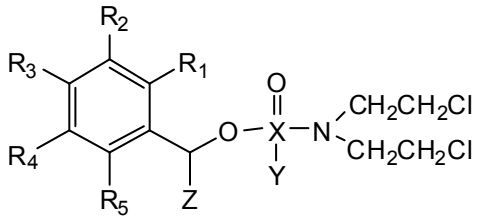


B



compound	**structure**	**diastereomer**
Compounds based on A		
LH3	X=O; Y=NH	*cis*
LH4	X=O; Y=NH	*trans*
LH5	X=NH; Y=O	*cis*
LH6	X=NH; Y=O	*trans*
LH12	X=NH; Y=NH	*cis*
LH13	X=NH;Y=NH	*trans*
Compounds based on B		
LH8	X=O; Y=NH	*cis*
LH9	X=O; Y=NH	*trans*

Table 2: Structure of acyclic nitrobenzylphosphoramide mustards.



compound	structure
LH7	R_3=NO_2; X=P; Y=NH_2; R_1=R_2=R_4=R_5=Z=H
LH14	R_3=NO_2; X=P; Y=NH_2; Z=CH_3; R_1=R_2=R_4=R_5=H
LH15	R_5=NO_2; X=P; Y=NH_2; R_1=R_2=R_3=R_4=Z=H
LH16	R_4=NO_2; X=P; Y=NH_2; R_1=R_2=R_3=R_5=Z=H
LH17	R_3=NO_2; R_5=OCH_3; X=P; Y=NH_2; R_1=R_2=R_4=Z=H
LH18	R_3=NO_2; R_4=OCH_3; X=P; Y=NH_2; R_1=R_2=R_5=Z=H
LH19	R_3=NO_2; R_4=CH_3; X=P; Y=NH_2; R_1=R_2=R_5=Z=H
LH24	R_3=NO_2; X=P; Y=NH_2; Z=CH_3; R_1=R_2= R_4=R_5=H
LH27	R_3=NO_2; X=C; R_1=R_2= R_4=R_5=Z=H
LH31	R_2=F; R_3=NO_2; X=P; Y=NH_2; R_1=R_4=R_5=Z=H
LH32	R_1=F; R_3=NO_2; X=P; Y=NH_2; R_2= R_4=R_5=Z=H
LH33	R_1=CF_3; R_3=NO_2; X=P; Y=NH_2; R_2= R_4=R_5=Z=H
LH34	R_1=Cl; R_3=NO_2; X=P; Y=NH_2; R_2= R_4=R_5=Z=H
LH37	R_1=R_5=F; R_3=NO_2; X=P; Y=NH_2; R_2= R_4=Z=H

Table 3: Substrate specificity of LmNTR. The apparent V_{max} and K_M values of HIS-tagged LmNTR toward various nitroaromatic and quinone-based substrates were determined in the presence of NADH (Material and Methods). The k_{cat}/K_M (the 'specificity constant') was then determined providing a useful ratio for comparing the relative rates of LmNTR activity on various substrates

compound	apparent K_M (μM)	apparent V_{max} (nmol min^{-1} mg^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Nitroimidazole			
benznidazole	22.0 ± 0.4	78.7 ± 1.0	2.5 x 10^3
metronidazole	2.4 ± 0.0	17.9 ± 0.0	5.2 x 10^3
Nitrofuran			
nifurtimox	9.7 ± 1.1	50.9 ± 7.0	3.7 x 10^3
nitrofurazone	4.7 ± 0.6	68.3 ± 10.0	1.0 x 10^4
nitrofurantoin	3.9 ± 0.5	56.7 ± 8.0	1.0 x 10^4
Nitrobenzyl			
CB1954	13.1 ± 0.1	85.9 ± 1.0	4.6 x 10^3
LH32	28.8 ± 2.7	100.7 ± 10.0	2.5 x 10^3
LH33	8.2 ± 1.0	114.2 ± 15.0	9.8 x 10^3
LH37	7.1 ± 0.4	71.7 ± 5.0	7.1 x 10^3
Quinone			
duroquinone	9.8 ± 0.3	140.6 ± 5.0	1.0 x 10^4
coenzyme Q1	5.1 ± 0.4	145.2 ± 11.0	2.0 x 10^4

Table 4: Susceptibility of *L. major* and differentiated THP-1 cells to nitrobenzyl phosphoramide mustards[a]

compounds	*L. major* IC_{50} (μM) promastigotes[b]	*L. major* IC_{50} (μM) amastigotes[c]	differentiated THP-1 IC_{50} (μM)[c]	selective toxicity[d]
nifurtimox	6.28 ± 0.04	2.15 ± 0.01	>100.00	>47
LH3-9 LH12-15; LH17-18; LH24	>30.00	>10.00	nd	nd
LH16	15.60 ± 1.13	2.75 ± 0.40	>100.00	>36
LH19	>30.00	4.72 ± 0.55	>100.00	>21
LH27	16.23 ± 0.29	4.65 ± 0.15	>100.00	>22
LH31	9.05 ± 0.56	7.00 ± 0.40	>100.00	>14
LH32	4.72 ± 0.23	1.09 ± 0.21	>100.00	>92
LH33	5.88 ± 0.28	0.21 ± 0.07	>100.00	>476
LH34	3.10 ± 0.28	0.77 ± 0.12	>100.00	>143
LH37	1.29 ± 0.08	2.17 ± 0.27	>100.00	>46

[a] nd, not determined. [b] Data are means from 4 experiments ± standard deviation. [c] Data are means from 3 experiments ± standard deviation. [d] The therapeutic index of a compound was calculated as a ratio of the IC_{50} against differentiated THP-1 cells to the IC_{50} against amastigote parasites.